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Inhibition of glucose-6-phosphate dehydrogenase protects hepatocytes from aluminum phosphide-induced toxicity

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ABSTRACT

Aluminum phosphide (AlP) poisoning is a severe toxicity with 30–70% mortality rate. However, several case reports presented AlP-poisoned patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency and extensive hemolysis who survived the toxicity. This brought to our mind that maybe G6PD deficiency could protect the patients from severe fatal poisoning by this pesticide. In this research, we investigated the protective effect of 6-aminonicotinamide (6-AN)- as a well-established inhibitor of the $NADP^+$- dependent enzyme 6-phosphogluconate dehydrogenase- on isolated rat hepatocytes in AlP poisoning. Hepatocytes were isolated by collagenase perfusion method and incubated into three different flasks: control, AlP, and 6-AN + ALP. Cellar parameters such as cell viability, reactive oxygen species (ROS) formation, mitochondria membrane potential collapse (MMP), lysosomal integrity, content of reduced (GSH) and oxidized glutathione (GSSG) and lipid peroxidation were assayed at intervals. All analyzed cellular parameters significantly decreased in the third group (6-AN + AlP) compared to the second group (AlP), showing the fact that G6PD deficiency induced by 6-AN had a significant protective effect on the hepatocytes. It was concluded that G6PD deficiency significantly reduced the hepatotoxicity of AlP. Future drugs with the power to induce such deficiency may be promising in treatment of AlP poisoning.

1. Introduction

Aluminum phosphide (AlP) is a pesticide used in many countries of the world. The pesticide is effective and cheap and very popular with the farmers [1]. Toxicity occurs due to the ingestion of AlP and injury from phosphine inhalation or even through the skin. After ingestion, AlP releases phosphine gas in the stomach in the presence of HCl, which is rapidly absorbed throughout the gastrointestinal tract, leading to systemic toxic effects involving the heart, kidney, liver, lung with manifestation of serious cardiac arrhythmias, intractable shock, acidosis and pulmonary edema [2]. The mechanism of AIP toxicity includes failure of cellular respiration due to the effect on mitochondria, inhibition of cytochrome C oxidase, formation of highly reactive hydroxyl radicals and cellular injury due to lipid peroxidation [3,4]. There is an increase in the activity of superoxide dismutase and decrease in the level of catalase in patients of AlP poisoning. The reduction of glutathione concentration in different tissues in AlP poisoning also explains the cellular injury. Interference with the function of the cellular enzymes and proteins is the main mechanism of toxicity [5]. It has been claimed that electron transfer and cytochrome C oxidase are blocked inhibiting oxidative phosphorylation and cellular respiration which finally activate peroxide radicals. Production of activated peroxide radicals results in further cell injury, afterwards. Other possible mechanisms of injury are inhibition of catalase and depletion of glutathione resulting in cellular dysfunction [2].

Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme in the pentose phosphate pathway which produces nicotinamide adenine dinucleotide phosphate (NADPH) to maintain an adequate reducing environment in the cells and is especially important in red blood cells (RBC). In addition to central role of G6PD in the regulation of redox

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state, it is understandable that mutations in the gene encoding G6PD can cause deficiency of the protein activity leading to neonatal jaundice and acute hemolytic anemia [6].

Despite such high mortality rate of AlP poisoning, there are several articles reporting glucose-6-phosphate dehydrogenase (G6PD) deficient patients exposed to AlP who survived [7]. Farnaghi and associates reported a case of G6PD deficiency who had attempted suicide by ingestion of AlP tablets. The patient experienced a severe course of toxicity including severe hemolysis but surprisingly survived the toxicity although it was too severe to be treated by the routine conservative therapies generally given to these patients [7]. This was a hint for us bringing the idea into our mind that probably G6PD deficiency had a protective role against AlP poisoning.

We checked the protective effect of G6PD deficiency induced by 6-aminonicotinamide (6-AN) in Sprague Dawley rat hepatocytes exposed to AlP. Cellular parameters such as viability, reactive oxygen species (ROS) formation, mitochondrial collapse (MMP), lysosomal integrity, content of reduced (GSH) and oxidized glutathione (GSSG), and lipid peroxidation were investigated.

2. Materials and methods

2.1. Chemicals

Aluminum phosphide (56%; Shanghai Ruizheng Chemical Technology Co., Shanghai, China), collagenase (Sigma-Aldrich company, Taufkrichen, Germany), piperazine-N0- (2-ethanesulfonicacid) (HEPES; Sigmachem Corporations), 70- dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich; Taufkrichen, Germany), Trypan blue (Sigma-Aldrich, Taufkrichen, Germany), and heparin (Zist Daru Darman Pars; Tehran, Iran) were used. All other chemicals were in the highest commercial grade available.

2.2. Animals

Male Sprague Dawley rats (150 to 200 g) were used for the study. They were purchased from Pasteur institute in Tehran, Iran, and fed with a standard chow diet and water. The experiments were performed according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran and in accordance with the “European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes” Acts of 1986 [8].

2.3. Isolation and incubation of hepatocytes

Rat hepatocytes were obtained by collagenase perfusion of the liver and the viability was assessed by plasma membrane disruption determined by trypan blue (0.2 w/v) exclusion test. Cells were suspended at a density of 10^6 Cells/mL in round-bottomed flasks rotating in a water bath maintained at 37 °C in Krebs-Henseleit buffer (pH = 7.4), supplemented with 12.5 mM HEPES under an atmosphere of 10% O_2, 85% N_2 and 5% CO_2. Each flask contained 10 mL of hepatocyte suspension. Hepatocytes were pre-incubated for 30 min prior to addition of chemicals [9]. To avoid either nontoxic or very toxic conditions in this study we used EC50 (2 h) concentration for ALP in the isolated hepatocytes (3 μg/mL). The EC50 of a chemical in hepatocyte cytotoxicity assessment technique (with the total 3 h incubation period), is defined as the concentration which decreases the hepatocyte viability to 50% following 2 h of incubation. In order to determine this value for ALP, dose–response curves were plotted and the EC50 determination was based on a regression plot of four different concentrations (Fig. 1 A). For the best protective effect of 6-AN against ALP induced hepatocytes toxicity and also to avoid toxic condition, we used IC50 concentration for 6-AN in the isolated hepatocytes (10 nM). The IC50 of 6-AN is defined as the concentration which decreases the G6PD activity down to

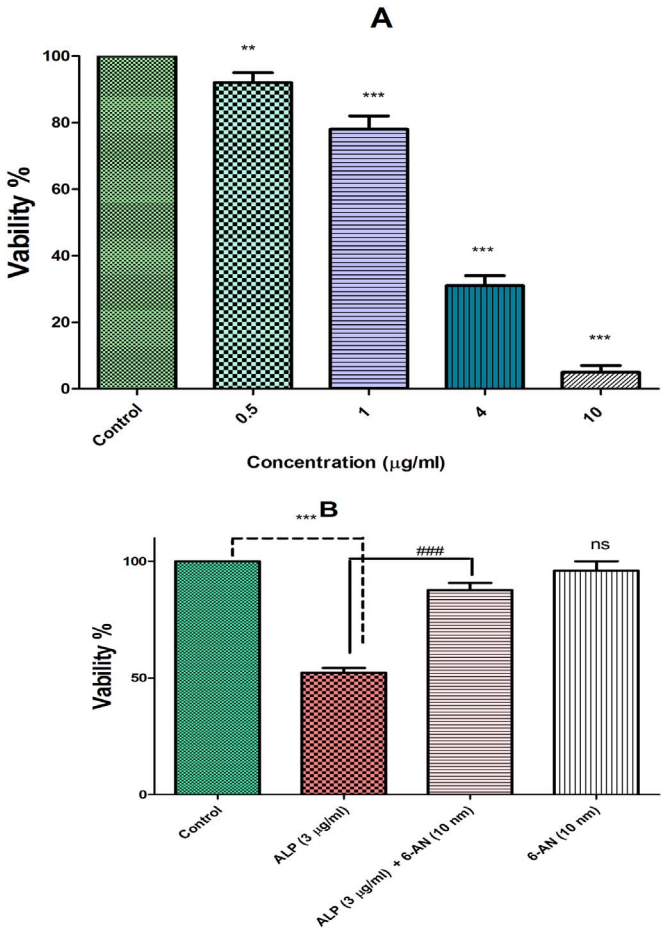

Fig. 1. Cell viability (A) and protective effect of 6-AN against cytotoxicity of ALP (B). Hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer with pH of 7.4 at 37 °C for 3 h. Cytotoxicity was determined as % cells that took up trypan blue. Values are expressed as mean ± SD of three separate experiments (n = 5).
The significant level was $P < 0.05$, n = 5
** And *** indicate significant ($P < 0.01$) and ($P < 0.001$) with untreated control respectively.
indicates significant ($P < 0.001$) with 6-AN group.
ns indicates non-significant with control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

50% following i0 min of pre incubation. 10 min is usually considered as pretreatment time in hepatocyte cytotoxicity assessment technique. In order to determine this value for 6-AN, dose–response curves were plotted and the IC50 determination was based on a regression plot of five different concentrations (Fig. 2 A).

2.4. Cell viability

The viability of isolated hepatocytes with and without of ALP (3 μg/mL) and 6-AN (10 nM) was assessed by evaluation of the intactness of the plasma membrane as determined by the trypan blue (0.2% w/v) exclusion test. Aliquots of the hepatocyte incubate were taken at different time points during the 3-h incubation period. At least, 80–90% of the control cells were still viable after 3 h [9].

2.5. G6PD deficiency

Dimethyl sulfoxide was used to prepare 6-AN solution. 10 nM of 6-AN was exposed to the hepatocytes in 37 °C for 10 min. The hepatocytes were then checked for G6PD deficiency. G6PD activity was checked by detection of the NADP + reduction to fluorescent NADPH using a fluorescent detector in 340- to 460- nm wavelengths.

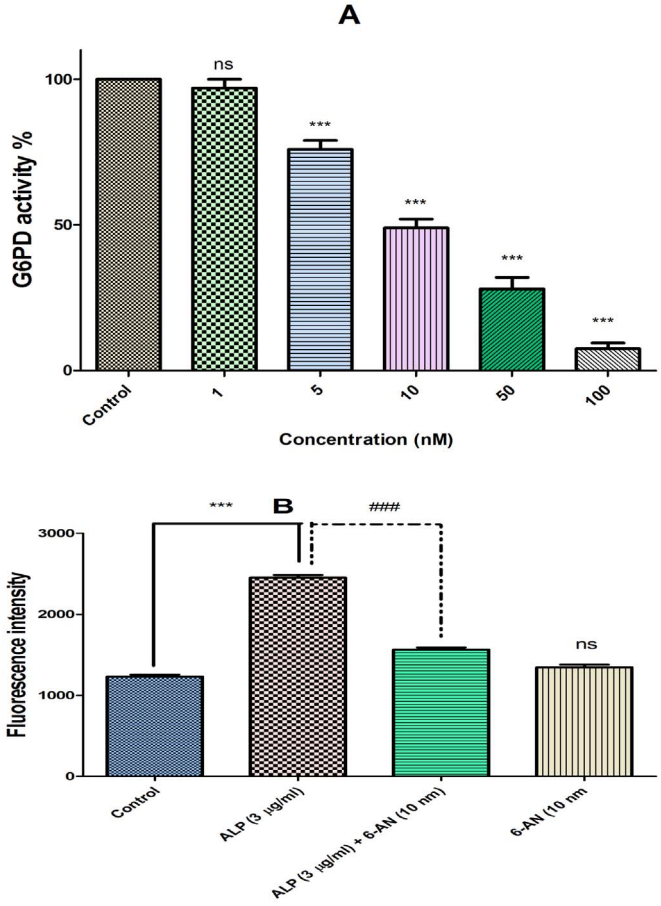

Fig. 2. G6PD activity (A) and ROS formation (B) in hepatocytes. G6PD activity and ROS formation was measured fuorometrically as described in materials and methods. Values are presented as fluorescence intensity (n = 5).
Data presented as mean ± SD
The significant level was $P < 0.05$
*** indicates significant ($P < 0.001$) with untreated control.
indicates significant ($P < 0.001$) with 6-AN group.
ns indicates non-significant with control.

2.6. Determination of ROS

To determine the rate of hepatocyte reactive oxygen species (ROS) generation, dichlorofluorescein diacetate (DCFH-DA, 1.6 µM) was added to the hepatocytes. It penetrates hepatocyte cells and becomes hydrolyzed to non-fluorescent dichlorofluorescein (DCFH). The latter then reacts with reactive oxygen species (ROS) to form the highly fluorescent dichlorofluorescein (DCF), which effluxes the cell. The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 nm and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells [10].

2.7. Mitochondrial membrane potential assay

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123 (1.5 µM), has been used for estimation of the mitochondrial membrane potential. The amount of rhodamine 123 remaining in the incubation medium was measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths [11].

2.8. Lysosomal membrane integrity assay

Briefly, 500 µl of the cell suspension previously stained with acridine orange (5 µM) was separated from the incubation medium by 1 min centrifugation at 1000 rpm. The cell pellet was then re-suspended in 2 mL of fresh incubation medium. Acridine orange redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths [11].

2.9. GSH and GSSG

GSH and GSSG after 3 h of exposure were measured by spectrofluorometric method. Briefly, 500 µl of the cell suspension was separated from the incubation medium by 1 min centrifugation at 1000 rpm. The cell pellet was then re-suspended in 2 mL of fresh incubation medium. GSH and GSSG were determined according to the spectrofluorometric method. Each sample was measured in quartz cuvettes using a fluorimeter set at 350 nm excitation and 420 nm emission wavelengths [12].

2.10. Lipid peroxidation

Evaluation of lipid peroxidation in hepatocyte was conducted by determining the amount of thiobarbituric acid reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU-7 spectrophotometer [11].

2.11. Statistical analysis

The three groups of the cells in three different flasks were compared using one-way ANOVA test. To evaluate which groups were significantly correlated, post hoc test was applied. The data was analyzed using Graph pad prism version 5. A P value < 0.05 was considered to be statically significant.

3. Results

3.1. Viability

ALP was tested against rat hepatocyte and showed significant decrease in cell viability in a concentration-dependent manner. 3-h EC50 concentration of ALP (3 µg/mL) was determined on isolated hepatocyte (Fig.1 A). On the basis of our cytotoxicity study, EC50 2 h concentration of ALP (3 µg/mL) was selected for evaluation of protective activity of 6-AN (10 nM). Protective activity of 6-AN was then determined on isolated rat hepatocyte. As shown in Fig. 1 B, the percentage of live cells was 48.27% versus 82.35% in the ALP- and 6-AN + ALP-treated groups, respectively, which prevented from ALP toxicity in hepatocytes.

3.2. G6PD deficiency

G6PD activity was checked by detection of the $NADP^+$ reduction to fluorescent NADPH using a fluorescent detector in 340- to 460-nm wavelengths. A significant decrease in G6PD activity was observed when hepatocytes were treated with 6-AN within 10 min (Fig. 2 A).

3.3. ROS formation

Incubation of hepatocytes with ALP (3 µg/mL) also caused a significant rise in ROS formation as compared with control hepatocytes ($P < 0.05$). Pretreatment of hepatocytes by 6-AN (10 nM) showed protection against AlP- induced ROS formation (Fig. 2 B).

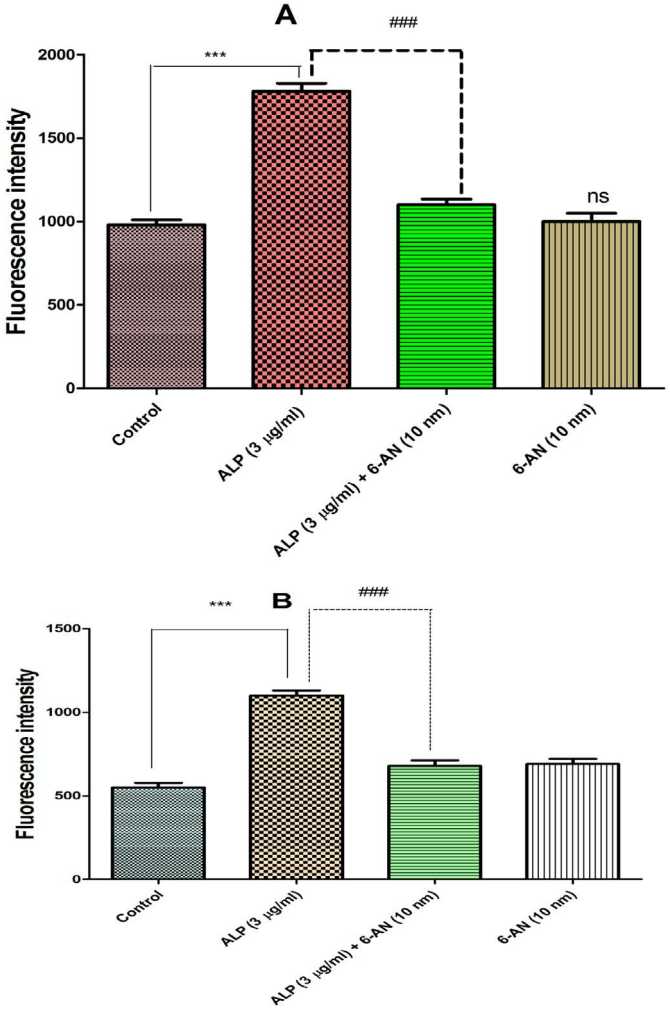

Fig. 3. MMP assay (A) and lysosomal integrity (B) in hepatocytes. ALP treated compared to those of untreated control. Collapse of MMP and lysosomal integrity were measured by rhodamine 123 and acridine orange respectively as described in materials and methods. Values represented as fluorescence intensity (n = 5).
Data presented as mean ± SD
The significant level was $P < 0.05$
*** indicates significant ($P < 0.001$) with untreated control.
indicates significant ($P < 0.001$) with 6-AN group.
ns indicates non-significant with control.

3.4. MMP collapse

To monitor the mitochondrial membrane potential, mitochondria were stained with rhodamine 123. Treatment of hepatocytes with AlP led to an increase in the mitochondrial collapse relative the control. Also, as shown in Fig. 3A, ALP (3 µg/mL) caused a rapid decline in mitochondrial membrane potential which was prevented by 6-AN (10 nM) (Fig. 3 A).

3.5. Lysosomal integrity

ALP (3 µg/mL) induced acridine orange redistribution in acridine orange-loaded rat hepatocytes, which is a marker of significant damage to lysosomal membranes. This cytotoxicity marker was also significantly prevented ($P < 0.05$) by 6-AN (10 nM) (Fig.3 B).

3.6. Lipid peroxidation

The ability of AlP to cause lipid peroxidation in hepatocytes was determined by measuring malondialdehyde (MDA) formation. As shown in Fig. 4 A, significant MDA formation could be observed when hepatocytes were treated with 3 µg/mL of AIP. This cytotoxicity marker was also significantly prevented ($P < 0.05$) by 6-AN (10 nM).

3.7. GSH and GSSG

As shown in Fig. 4, reduced (GSH) and oxidized (GSSG) glutathione levels were determined under the same experimental conditions and correlated with MDA formation and other measured parameters. A significant decrease in reduced glutathione (GSH) level was observed when hepatocytes were treated with 3 µg/mL of AlP within 3 h followed by an increase in oxidized (GSSG) glutathione levels. These cytotoxicity markers were also significantly prevented ($P < 0.05$) by 6-AN (10 nM) (Fig. 4 B and C).

4. Discussion

There is a clear evidence that a chronic decrease in metabolic rate results in AlP resistance [13], whereas an increase in metabolic demand causes hypersensitivity toward AlP [14]. There are three very different toxicity mechanism for AlP including inhibition of the acetylcholine esterase, interaction of AlP with enzymes involved in metabolic processes, and oxidative stress, all of which, are potentially interrelated mechanisms metabolic alteration in the cell [15]. The first mechanism is signaling through acetylcholine, which is both an excitatory neurotransmitter and the primary regulator of metabolism through the parasympathetic nervous system. Previous studies have shown that the muscarinic acetylcholine antagonist- atropine- protects rats against AlP exposure indicating that acetylcholine signaling is an important component of AlP toxicity [16].

Altered metabolism is a key in AlP toxicity, but this may either be a direct effect as through induction of a metabolic crisis or an indirect effect through an increase in ROS generation or some other metabolic processes [15]. Aluminum phosphide acts at complex IV of the electron transport chain to inhibit electron flow through the electron transfer chain (ETC), though the AlP effect is more significant in vitro than in vivo [16,17]. Glycerophosphate dehydrogenase is inhibited by AlP which results in stimulation of hydrogen generation [18]. It has been noted that fumigation induces superoxide dismutase but inhibits peroxidase and catalase activity [19–21]. Our results showed that 6-AN protected against oxidative stress and decreased AlP toxicity in rat hepatocytes (Fig. 2). Aluminum phosphide-induced generation of ROS provides a strong alternative model to mortality caused by energy insufficiency. The decrease in ROS generation associated with decreased energy metabolism may explain how 6-AN can enhance tolerance to AlP.

Aluminum phosphide inhibits respiration in rat liver mitochondria, insect mitochondria, and intact nematodes [17,19,22,23]. Our results showed that AlP exposure to hepatocytes led to mitochondria collapse while interestingly mitochondrial damages decreased in presence of 6-AN as a G6PD inhibitor (Fig. 3). However, careful analysis using specific substrates revealed complex IV, cytochrome C oxidase, to be the primary site of in vitro interaction between AlP and the electron transfer chain [17,21,23]. As AlP is known to inhibit cytochrome C oxidase, and AlP-induced generation of ROS. Disruption of mitochondrial respiration by AlP suggested that energy insufficiency was the cause of mortality due to AlP exposure [24].

Glutathione protected against oxidative damage and enhanced cell survival. Previous studies showed that endogenous glutathione levels did not change over the treatment period and that glutathione was likely acting as an antioxidant as it did not inactivate the AlP directly [25,26]. While in this study we showed that reduced glutathione decreased and oxidized glutathione increased, finally glutathione depletion inhibited by 6-AN, the results are supported by observations that treating rats or nematodes with glutathione depleting chemicals results in increased sensitivity to AlP [14,27].

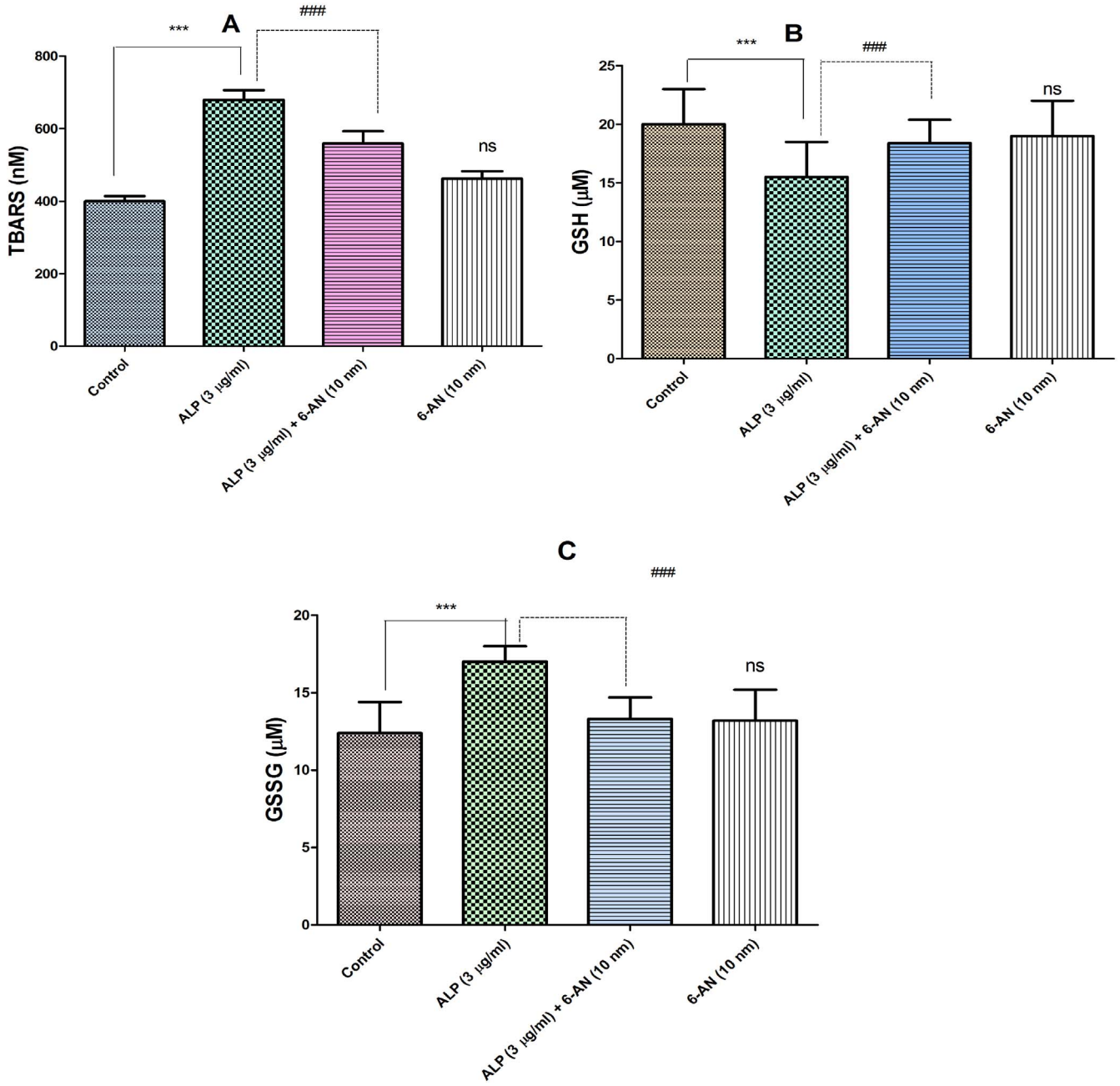

Fig. 4. Hepatocytes (10^6 cells/mL) were incubated at 37 °C for 1.5 h following the addition of ALP and 6-AN. TBARS formation was expressed as nM concentrations (C). Also intracellular GSH (B) and extra cellular GSSG (C) were fluorimetrically determined.
Data presented as mean ± SD
The significant level was $P < 0.05$
*** indicates significant ($P < 0.001$) with untreated control.
indicates significant ($P < 0.001$) with 6-AN group.
ns indicates non-significant with control.

We showed that G6PD induced by 6-AN could protect the hepatocytes from damage when exposed to AlP. ROS formation, mitochondrial damage, lysosomal damage, and lipid peroxidation were also decreased when deficiency was induced in the hepatocyte G6PD enzymatic activity confirming its protective role against PH_3 exposure. Further studies on the drugs that can induce such deficiency in the human beings and protect them from AlP poisoning are guaranteed. However, it should be borne in mind that the induction of G6PD deficiency –as a therapeutic intervention- should not be too severe to threaten the patients' life, per se.

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